REMARKS

Entry of the foregoing, reexamination and reconsideration of the above-identified application are respectfully requested.

Applicants note with appreciation the courtesies extended to applicants and their representatives during the interview of November 7, 2003. During the interview, all of the claims were discussed. The Examiner suggested amending the claims to recite an "edible-oil composition" as now recited in the claims, using "product-by-process" language. The process for producing the composition is also recited in the claims. Support for these claims is found in the application and the prior claims. No new matter is added by this amendment. Applicants reserve the right to pursue the prior claims in additional application(s).

Claims 13, 14, 29, 30, 32-36 and 47-60 have been rejected under 35 U.S.C. §103(a) as allegedly being unpatentable over Shinmen et al in view of both Shimizu et al and Barclay. This rejection is respectfully traversed.

Shinmen is said to teach that an unsaturated fatty acid-containing oil contains about 18-60% arachidonic acid. Shimizu et al is also said to teach that unsaturated fatty acid-containing oil obtained from culturing microorganism *Mortierella* has 24,25-methylenecholest-5-en-3β-ol, which has not been found in nature, but does not teach how much is present in the oil. Barclay is cited as teaching the employment of arachidonic acid containing oil for food products, such as baby and animal food. It allegedly would have been obvious to modify the unsaturated fatty acid-containing oil of Shinmen et al by

removing the biologically unknown compound, i.e., 24,25-methylenecholest-5-en-3β-ol, and to employ the modified oil in food products, e.g., baby food, animal food or nutritive dietary supplement, since the biological activity was not known.

It is respectfully submitted that the instantly claimed invention would not be obvious in view of the cited art. The cited art could not be combined as proposed in the Official Action, to obtain the instant invention as claimed.

The instant invention is directed to an edible-oil composition produced by culturing a producer microorganism belonging to the genus *Mortierella* in a fermentor with aeration in the presence of a nitrogen source. Such an edible-oil composition will have a very low ratio of 24,25-methylenecholest-5-en-3β-ol to desmosterol, e.g., a 24,25-methylenecholest-5-en-3β-ol compositional ratio in a proportion of 1.2 or less with respect to the desmosterol compositional ratio, an arachidonic acid content of 30 to 50%, and a 24,25-methylenecholest-5-en-3β-ol compositional ratio of 35% or lower. Such an edible-oil composition could not have been obtained by removing the 24,25-methylenecholest-5-en-3β-ol. At the time of the invention, there was no known method for separating the 24,25-methylenecholest-5-en-3β-ol to obtain such a low ratio. This is evidenced by the Akimoto Declaration Under 37 C.F.R. §1.132, submitted herewith.

As stated by Dr. Akimoto in Paragraph 1, "[t]he inventive feature of the above-mentioned patent application is to obtain the microbial oil containing poly-unsaturated fatty acids with less 24,25-methylenechorelst- 5-en-3beta-ol content obtainable by fermentation of strains belonging to Mortierella subspecies of Mortierella species in a fermentation

medium containing nitrogen source derived from soybean." These strains accumulate significant amount of oil (triglycerides) containing poly-unsaturated fatty acids as their constituting fatty acids, which is obtained by extraction with hexane. They produce 24,25-methylenechorelst- 5-en-3beta-ol (Component A) as well as desmosterol (Component B) simultaneously, which also exist in the oil extracted. As recognized by Dr. Akimoto, the goal is "to reduce the amount of Component A as much as possible, for it is an unfavorable component of little to none eating habit whereas we have no worry about Component B, which is a component in human breast milk and thus within our eating habit." The instant invention was thus directed to making the proportional content of Component B higher compared with A to achieve the reduction of Component A. Surprisingly, the inventors found that this goal could be achieved by fermenting the strains in the medium containing nitrogen source derived from soybean.

Component A and Component B are both sterols naturally existing in the microbial oil (triglycerides) as unsaponified matter. Sterols may be reduced to a certain amount by conventional oil refining process (degumming, deacidification, bleaching, and deodorization), which is generally applied to edible plant oil/fat production. There are many reports on the sterol content obtained during the refining process. Johansson et al, *Journal of the American Oil Chemists' Society*, 56(10):883-889 (1979), for example, sets forth the sterol content (mg/g) in soybean oil in Table 1 as follows:

Table 1 Sterol content after the conventional oil refining process				
	Process I		Process II	
Process	Free-type	Ester-type	Free-type	Ester-type
Degumming	3.1	0.6	3.4	0.6
Deacidification	3.0	0.6	3.0	0.6
Bleaching	1.8	0.5	2.0	0.6
Deodorization	1.8	0.5	1.6	0.6

As explained by Dr. Akimoto in his Declaration at Paragraph 7, sterols are classified in two types; free-type and ester-type. As seen from Table 1, free-type sterols are the ones removed during the conventional oil refining process. By contrast, the content of ester-type sterols remains unchanged, as shown in Table 1. Even free-type sterols may not be removed completely during the refining process. It is, therefore, important to find a way to reduce the sterol content of Component A in the extracted oil *before* the refining process in view of the fact that the sterol content cannot be completely removed during the refining process.

Elimination of 24,25-methylenecholest-5-en-3β-ol present in a fatty acid-containing product is *very* difficult. The difficulty is due to the fact that the polarity of the 24,25-methylenecholest-5-en-3β-ol and of oil contained in a fermentation broth are substantially the same. Therefore, conventional purification methods cannot be used to simply remove the 24,25-methylenecholest-5-en-3β-ol from the oil. Applicants are not aware of *any*

references describing a method for removing the 24,25-methylenecholest-5-en-3β-ol from oil or lipid.

In the instant specification, Examples 1-3 describe the ratio of 24,25-methylenecholest-5-en-3β-ol to desmosterol in a hexane extract, and Example 4 describes the same ratio in oil after purification. First, it should be noted that substantially all the lipid components are extracted by hexane; therefore, the ratio in lipid in a fermentation broth before the extraction and the ratio in the hexane extract are substantially the same.

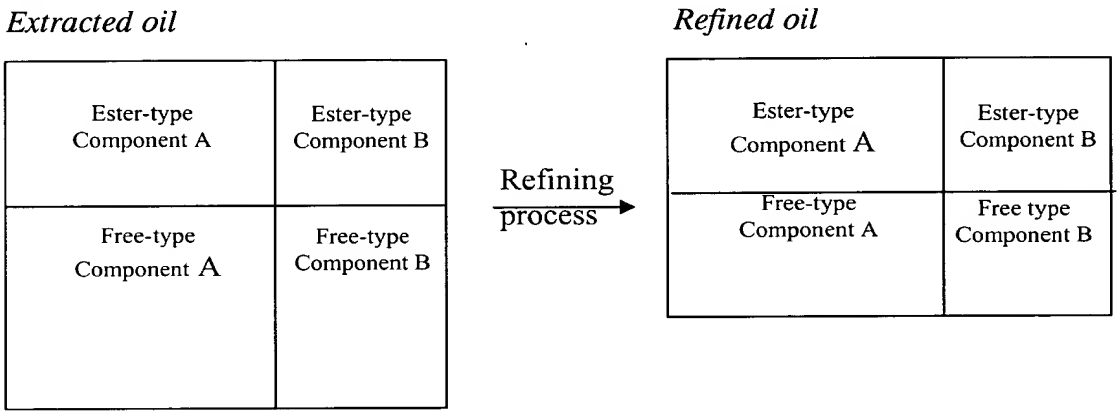
According to Example 1, the 24,25-methylenecholest-5-en-3β-ol content is 30% and the desmosterol content is 66% to give a ratio of 0.46. According to Example 2, the 24,25-methylenecholest-5-en-3β-ol is 25% and the desmosterol content is 53%, to give a ratio of 0.47. According to Example 3, the 24,25-methylenecholest-5-en-3β-ol content is 5% and the desmosterol content is 67% or 35%, to give a ratio of 0.07 or 0.14, respectively. In Example 4, the 24,25-methylenecholest-5-en-3β-ol content is 24% and the desmosterol content is 67%, to give a ratio of 0.35.

It is clear that a conventional purification process as described in Example 4 cannot change the ratio of the 24,25-methylenecholest-5-en-3β-ol to desmosterol content. Thus, to obtain a final lipid or oil product having the ratio of 24,25-methylenecholest-5-en-3β-ol to desmosterol content within the claimed ratio, it is essential to obtain a crude lipid or oil in a fermentation broth in which the ratio in that crude lipid or oil is within the claimed ratio. How to obtain such a lipid or oil is no where taught or suggested in the cited references.

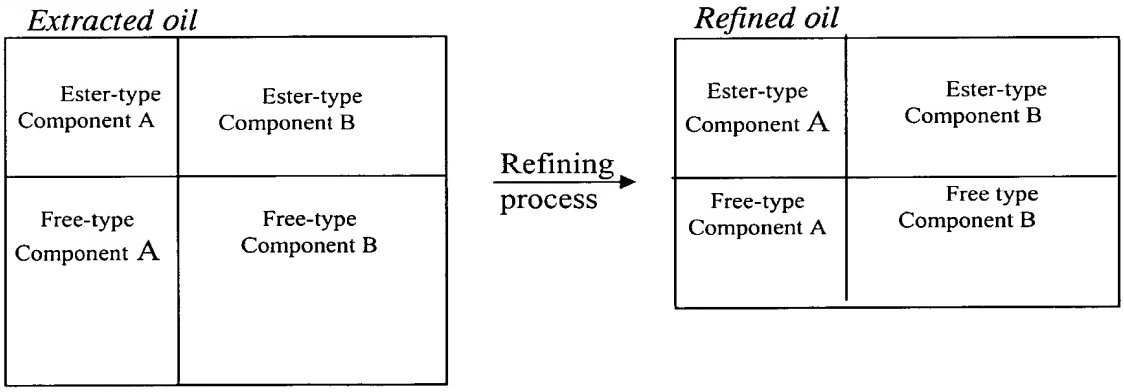
Without a means in the art for obtaining the oil as claimed, the product itself cannot have been known or obvious prior to Applicant's invention.

A chart is provided in Dr. Akimoto's Declaration in Paragraph 8. This shows the difference in the oil compositions of the prior art and the instant invention, as follows:

Prior art:



Present Invention:



The extracted oils naturally contain Ester-type Component A, Free-type Component A, Ester-type Component B, and Free-type Component B. As can be seen from the chart, the ester-type sterols remain unchanged during the refining process whereas free-type

sterols are not completely removed by the refining process. The refined oil consequently contains Ester-type Component A and Ester-type Component B, both unchanged, and Free-type Component A and Free-type Component B, both reduced by still present after the refining process. As stated by Dr. Akimoto in Paragraph 10:

> In the end in order to obtain the oil with less Component A, which we have little eating habit with, it is important to obtain the oil with more content of Component B, and we have achieved the goal by fermenting the strains belonging to Mortierella subspecies of Mortierella species in a fermentation medium containing nitrogen source derived from soybean.

The Official Action dated April 9, 2003, asserted on page 3:

> Therefore, it would have been prima facie obvious to a person of ordinary skill in the art, at the time the claimed invention was made, to modify the unsaturated fatty acid-containing oil of Shinmen et al. by *removing* the biologically unknown compound, i.e., 24,25-methylenecholest-5-en-3β-ol and employ the modified oil in food products such as baby food and animal food or in nutritive dietary supplement.

This assertion is in error. As shown *supra*, the 24,25-methylenecholest-5-en-3β-ol cannot be removed using standard refining processes to produce an arachidonic acid-containing oil in accordance with the claimed invention. The instant invention, by reducing the amount of 24,25-methylenecholest-5-en-3β-ol obtained during production, thus overcomes the problem in the art of how to reduce the 24,25-methylenecholest-5-en-3β-ol

content in products such as baby food, animal food and nutritive dietary supplements. Prior to the instant invention, an edible oil having a low content of 24,25-methylenecholest-5-en-3β-ol in proportion to the desmosterol could not have been obtained.

Thus, by combining the references as proposed in the Official Action, the claimed invention is not obtained. Withdrawal of the prior art rejection of record is respectfully requested. Such action is believed to be in order.

In view of the above, it is respectfully submitted that the claimed invention is not disclosed by, nor rendered obvious by the cited art. Withdrawal of the rejection of record is respectfully submitted and believed to be in order.

It is respectfully submitted that the application is now in condition for allowance. Thus, a Notice of Allowance is respectfully requested.

In the event that there are any questions relating to this amendment or the application in general, it would be appreciated if the Examiner would contact the undersigned attorney by telephone at (650) 622-2360 so that prosecution of the application may be expedited.

Respectfully submitted,

BURNS, DOANE, SWECKER & MATHIS, L.L.P.

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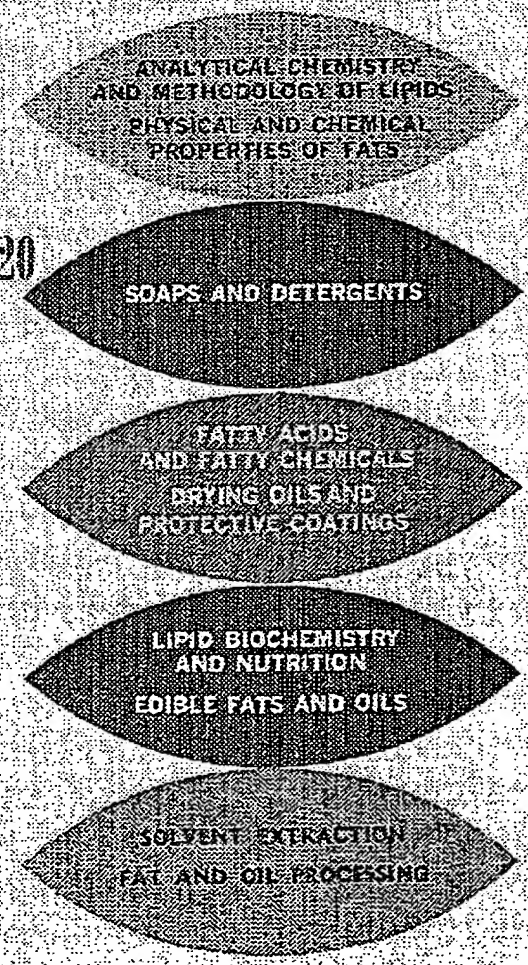

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The Effect of Processing on the Content and Composition of Free Sterols and Sterol Esters in Soybean Oil

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ABSTRACT

The content and composition of free sterols and sterol esters in crude soybean oil and in oils from different stages of two continuous refining systems were determined. The sterols were isolated by preparative thin layer chromatography and analyzed by gas chromatography with cholesterol as an internal standard. The free sterols in one of the degummed oils amounted to 3.1 mg/g and were diminished to 1.8 mg/g oil by the De Laval Short-Mix refining process. The content of free sterols of the other degummed oil was reduced from 3.4 to 1.6 mg/g oil by the Zenith process. The greatest reduction of sterol content was caused by the treatment with bleaching earth. The sterol esters accounted for 0.6 mg/g of the degummed oil, and only very small changes were observed during the processes. However, changes in the composition of fatty acids of the sterol esters were found. These changes might indicate a selective deacylation of sterol esters or an interesterification during the refining processes. The composition of sterols in free and esterified form were different. Campesterol, stigmasterol and sitosterol were obtained in both free and esterified form, but Δ7 stigmasterol was only found in esterified form. Only small changes in the percentage distribution of the sterols occurred during the processes.

INTRODUCTION

Sterols exist in crude soybean oil as free sterols, sterol esters, sterol glucosides and acetylated sterol glucosides (1,2). However, no reports seem to present data on sterol glucosides and acetylated sterol glucosides in degummed oils, and considering the high polarity of these compounds they are likely to be removed during the degumming process. Furthermore the "lecithin gums" are reported to contain rather high amounts of sterol glucosides (3). Sterols are removed during the refining processes. This makes the soapstock and the deodorizer distillate a source for sterols as raw materials in chemical industries (4-7). Sterols are generally regarded as heat-stable as well as odorless and tasteless (8), making them of less interest as regarding the oil quality. However, several investigations have shown that treatment with bleaching earth causes the formation of sterol "artifacts" and that sterol esters are deacylated (9-12).

Sterols are usually obtained after saponification of the oil, and the sterol pattern is used to characterize the oil and to detect adulterations (4,13).

This paper presents data on the content and composition of free sterols and sterol esters, obtained in degummed, neutralized, bleached and deodorized soybean oils. Two continuous refining processes, the De Laval Short-Mix process and the Zenith process, were studied.

MATERIAL AND METHODS

Material

Samples of degummed, neutralized, bleached and deodorized soybean oils were obtained from the De Laval Short-Mix process (Refining system I) (8) and from the Zenith process (Refining system II) (14). The degummed oil was neutralized at 92-95 C with 2.5 M sodium hydroxide in process I and with 0.35 M in process II. The bleaching procedures were similar in the two processes, using 1.5% bleaching earth (Tonsil, Optimum Standard) at 95-105 C. In process I the deodorization was performed at 190 C for 5 hr using a batch procedure, while process II was a continuous procedure (The Girdler Process) operating at 230 C for 1 hr.

Solvents used in the sterol analyses were of "pro analysi" grade from Merck. Cholesterol, TLC reference mixture No. 1 and AOCS No. 11, reference mixture for GLC, were purchased from Nu Check Prep., Inc., Elysian, MN, USA. Thin layer plates, silica gel 60, 20 x 20 cm, 0.5 mm from Merck were used while other chromatographic materials were obtained from Analabs, Inc., North Haven, CT, USA.

Thin Layer Chromatography

The free sterols and sterol esters were isolated from the oil samples by preparative TLC. A known amount (ca. 20 mg) of the oil and a reference mixture (TLC No. 1) were applied to each of three thin layer plates. Hexane/diethyl ether/acetic acid (70:30:1 v/v) was used as the developing solvent. After development, the reference lane was sprayed with dichlorofluorscein (0.025% in ethanol). The sterols

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TABLE I

Free and Esterified Sterols in Soybean Oil after Different Processing Steps

	Refining process I		Refining process II	
	Free sterols (mg/g oil)	Esterified sterols (mg/g oil)	Free sterols (mg/g oil)	Esterified sterols (mg/g oil)
Degummed	3.1	0.6	3.4	0.6
Neutralized	3.0	0.6	3.0	0.6
Bleached	1.8	0.5	2.0	0.6
Deodorized	1.8	0.5	1.6	0.6

and sterol esters of the sample were located, scraped from the plate, and eluted from the silica gel with diethyl ether. To both the free and esterified sterols ca. 10% of cholesterol was added as an internal standard. Separate analyses of degummed and bleached oil without the addition of internal standard were also performed.

The sterol esters were hydrolyzed, and then the sterols and the fatty acids were extracted and separated as previously described (15).

Gas Chromatography

The sterols were silylated (15) and analyzed by GLC in a Varian Aerograph Model 2100 equipped with two glass columns (180 cm x 2 mm) packed with 1% OV 17 on HP Chromosorb G 80/100 mesh and 3% SE-30 on Var-a-port 100/120 mesh and kept at 270 C and 250 C, respectively. The sterol composition was calculated from duplicate analyses on OV-17, while chromatograms from the SE-30 phase were examined to detect possible peak overlapping. The TMS sterols were identified by comparison of the retention times of actual TMS sterols with known samples.

The reproducibility of the qualitative and quantitative measurements were studied in two subsequent analyses of two sterol samples.

Fatty acid methyl esters were prepared and analyzed as described previously (15), but with a column temperature programmed from 140 to 190 C at the rate of 2 C/min. Corrections for contaminants which accumulated in a blank sample during the procedure were also made.

RESULTS

Sterol Content

The sterols in crude, degummed soybean oil appeared in a larger quantity in free (3.1 and 3.4 mg/g oil) than in esterified (0.6 mg/g oil) form (Table I). During the refining processes, the amount of free sterols was decreased while the content of esterified sterols was almost constant. The total loss of sterols was slightly higher in process II (45%) than in process I (38%).

In process I the neutralization had almost no effect on the sterol content (Table I), whereas 0.4 mg sterols/g oil were removed in process II, in which a more dilute alkaline solution (0.35 M vs. 2.5 M) was used. Treatment with bleaching earth caused the greatest loss of sterols (1.0 and 1.2 mg/g oil) during the processes. The deodorization in process I (190 C for 5 hr) seemed to have no effect on the sterol content, while during process II (230 C for 1 hr), 0.4 mg/g oil was removed.

Repeated isolations of the sterols gave figures not differing more than ± 0.1 mg/g oil.

Sterol Composition

Campesterol, stigmasterol and sitosterol appeared in both free and esterified form though in a different percentage distribution. The Δ7-sterols were preferentially present in esterified form. Traces of cholesterol esters were obtained in degummed and bleached oils, which were the only oils analyzed separately without addition of the internal standard.

The free sterols in degummed oil were composed of 52% of sitosterol, 25% of campesterol and 23% of stigmasterol (Table II). A selective removal, consisting of slightly more campesterol and stigmasterol than sitosterol, during the technological processes resulted in a somewhat different sterol composition in the refined oil compared to the crude oil.

Sitosterol (70-74%) predominated among the esterified sterols in degummed oil, followed by campesterol (ca. 12%), Δ7-stigmastenol (ca. 9%), stigmasterol (ca. 6%) and

TABLE II

Distributions of Free and Esterified Sterols in Soybean Oil after Different Processing Steps

	Refining process I					Refining process II				
	Campesterol	Stigmasterol	Sitosterol	Δ7-Stigmastenol	Δ7-Avenasterol	Campesterol	Stigmasterol	Sitosterol	Δ7-Stigmastenol	Δ7-Avenasterol
Free sterols										
Degummed[a]	25.4	22.5	52.1	tr	...	25.2	23.4	51.4	tr	...
Neutralized[a]	25.2	22.8	52.1	tr	...	25.1	23.1	51.7	tr	...
Bleached	24.4	22.3	53.3	...	...	24.8	20.7	54.6	tr	...
Deodorized (refined)	24.3	21.3	54.4	...	...	23.1	26.7	56.2	tr	...
Esterified sterols										
Degummed	12.8	6.8	69.9	9.5	1.0	11.8	6.0	73.6	7.6	1.0
Neutralized	10.7	10.5	66.3	12.5	tr	11.3	5.3	73.8	8.4	1.8
Bleached	10.3	5.0	76.0	8.7	tr	11.6	4.9	76.1	7.4	tr
Deodorized	12.4	6.4	73.4	7.8	tr	11.8	7.4	71.7	9.0	tr

[a]Traces of Δ5-avenasterol appeared among the free sterols in degummed and neutralized oil in processes I and II.

TABLE III

Compositions of Fatty Acids of Sterol Esters in Soybean Oil after Different Processing Steps

	Fatty acids (%)								
	16:0	18:0	18:1	18:2	18:3	20:0	22:0	24:0	Others[a]
Refining process I									
Degummed	6	3	10	16	2	3	12	11	37
Deodorized	6	5	7	27	4	6	21	19	5
Refining process II									
Degummed	5	3	9	19	2	3	14	12	33
Neutralized	7	4	9	23	2	3	16	14	22
Bleached	6	1	6	69	5	3	5	6	tr
Deodorized	12	7	13	45	3	3	9	8	tr

[a]The percentages in the crude oils consist of 1-2% of 16:1, 6-7% of 20:1, 4-5% of 22:1, 1-3% of 24:1 and five unidentified components. These were registered after 16:1 (3-4%), after 18:2 (6-7%), after 20:1 (4-6%), and after 22:1 (3% and 3%) at the chromatograms.

Δ7-avenasterol (1%) (Table II).

The sterol composition obtained in oils from the investigated refining stages showed some differences, the greatest being between neutralized and bleached oil in process I, with 66.3 vs. 76.0% of sitosterol, and between bleached and deodorized oil in process II, with 76.1 vs. 71.7% of sitosterol (Table (II). In both processes sitosterol was found in a lower percentage in the deodorized than in the bleached oil.

Repeated analyses of the sterol composition in two separate isolations showed, at the most, 0.5% differences.

Fatty Acids of Sterol Esters

The composition of the fatty acids of sterol esters was rather complex and no fatty acid predominated (Table III). The major fatty acids in the degummed oil, calculated after subtraction of the fatty acids in the blank sample, were 18:1 (9-10%), 18:2 (16-19%), 22:0 (12-14%) and 24:0 (11-12%).

The fatty acids obtained in oils from the consecutive stages of process II displayed a different composition (Table III). After neutralization the fatty acids marked "others" had decreased 11%, and in the bleached oil only traces could be detected. Very long chain fatty acids (C 22 and C 24) had also decreased during bleaching, while the percentage of 18:2 increased and amounted to 69% of the total fatty acids. During deodorization a decrease in percentage of polyunsaturated fatty acids (18:2 and 18:3) was found.

The deodorized oil from process I yielded a much lower percentage of 18:2 and a higher percentage of 22:0 and 24:0, compared to the oil from process II. Fatty acids of sterol esters were not analyzed in neutralized and bleached oil from process I.

DISCUSSION

The content of sterols in the crude, degummed oil was within the limits of literature data, which ranged form 3.4 to 4.2 mg/g oil (16-21). Japanese reports on free and esterified sterols show three to five times as much free sterols as esterified sterols (22,23), which agree with the present investigation. Bulgarian soy, however, yielded a greater difference between the content of free (3.0 mg/g) and esterified (0.1 mg/g) sterols (3).

The removal of sterols from oils during the refining is well established (7,8). The reported amount of sterols which are removed (25-35%) from soybean oil varies due to the technological processes used (4,5). The total sterol content as well as whether the sterols appear in free or esterified form is also likely to influence the percentage of sterols removed. Gutfinger and Letan (5) followed the quantitative changes in the consecutive refining stages and found that the greatest loss of sterols was during neutralization and deodorization. Others (24) have shown that during the deodorization free sterols are preferentially removed.

In the present investigation two continuous refining processes were studied. The impact of each individual parameter on the removal of sterols cannot be stated, but some comparisons between the two methods can be made. In the neutralization method in process II a more dilute alkaline solution was used. This probably resulted in a greater decrease in the sterol content, compared to the method in process I. No sterols seemed to be lost during deodorization at 190 C, while at the higher temperature (230 C) 0.4 mg of free sterols/g oil was removed.

The greatest loss of sterols in both processes occurred during the bleaching process. According to previous investigations (9-12), losses during treatment with bleaching earth are mainly due to the formation of nonpolar steroids and, to a smaller extent, to absorption to the bleaching earth. Studies on sterol esters of long chain fatty acids showed that they were deacylated during treatment with bleaching earth, and products similar to those obtained from free sterols were formed (11). Furthermore, the quantity of nonpolar steroids formed during treatment with bleaching earth was shown to be proportional to the quantity of esterified sterols (6), which more easily undergo dehydration than the free sterols. In the referred study, comparison between sunflower, with a relatively small content, and rapeseed oil, with larger content of sterol esters, was carried out. Soybean oil contains even smaller quantities of sterol esters than sunflower oil (25), and we observed little change in the content of sterol esters during bleaching. Besides, we have observed that the content of sterol esters in rapeseed oil is noticeably diminished during treatmeant with bleaching earth (Johansson, A., unpublished results). Although the content of sterol esters was almost constant during the refining processes, some changes in the composition of sterols and of fatty acids of sterol esters were found. Noticeable was the increase in percentage of 18:2, from 25% to 69%, during the bleaching procedure. The changes might be due to a selective deaclylation of sterol esters, which were too small to be observed in the quantification method used, or to an interesterification.

The total sterol composition agreed with most literature data, which show the following ranges: 18-24% of campesterol, 18-24% of stigmasterol and 53-59% of sitosterol (17, 19-21, 26-29). Δ5-Avenasterol (0-3%), Δ7-stigmastenol (0-5%) and Δ7-avenasterol (0-1%) have also been reported, and in two investigations as much as 2% of cholesterol was

found (27, 28). A notable lower percentage of stigmasterol (11-12%) balanced by a higher percentages of sitosterol and Δ5-avenasterol was reported in two investigations (30,31).

Δ7-Stigmastenol has not previously been reported in esterified form in soybean oil. The percentages of campesterol and stigmasterol were shown to be relatively lower and sitosterol higher in esterified than in the free form (24), which agrees with the present investigation. Since the ratio of esterified sterols compared to free sterols was greater in refined than in crude oil, the total percentage of stigmasterol was lower in the refined oils. When comparing the sterol compositions of different soybean oil, it is necessary, therefore, to state whether the oils are crude or refined.

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